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# Kinetics of Duck Hepatitis B Virus Infection Following Low Dose Virus Inoculation:



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Using pooled serum from congenitally duck hepatitis B virus (DHBV)-infected ducks as inoculum, we examined the effect of virus dose on the incubation period of infection and on the patterns of spread of virus infection in the liver. The pooled serum inoculum contained $9.5 \times 10^9$ DHBV genomes per milliliter and had an infectivity titre ($ID_{50}$) in newly hatched ducks of $1.5 \times 10^{10}$ per milliliter with a 95% confidence interval of $3.0 \times 10^9$ to $6.3 \times 10^{10}$ $ID_{50}$/ml, indicating the equivalence between one DHBV genome and one infectious unit within the limits of the assays. The incubation period of infection was inversely related to the dose of inoculum and the onset of viraemia ranged from Day 6 with the highest dose to Day 14 or 29 with the lowest dose inoculum. To study the spread of virus infection from a low percentage of initially infected cells we inoculated newly hatched ducks intravenously with sufficient DHBV ($1.5 \times 10^3$ $ID_{50}$) to infect only ~0.0001% of total liver cells. DHBV infection first reached detectable levels on Day 4 postinoculation (p.i.) and was detected in ~0.035% of hepatocytes, most of which occurred as single cells or pairs of cells, indicating that a number of rounds of infection had occurred with the spread of virus both to adjoining cells, i.e., by cell-to-cell spread, and to cells located in other parts of the liver lobule. Despite some bird-to-bird variation in timing, the percentage of infected hepatocytes increased exponentially with a mean doubling time of 16 hr from Day 4 to Day 14 p.i., by which time replication was seen in >95% of hepatocytes. This rapid dissemination from a small number of infected hepatocytes suggests that, in neonatal ducks, there are no major delays in virus replication within the liver, that any innate and adaptive defence mechanisms operating during the first 10 to 14 days of infection are insufficient to contain virus spread, and that even a small number of infected hepatocytes produce enough progeny to rapidly infect the remaining hepatocytes. 

## INTRODUCTION

The hepadnavirus family which includes the prototype human hepatitis B virus (HBV), as well as viruses specific to woodchucks, ground squirrels, herons and ducks (duck hepatitis B virus; DHBV), are small, enveloped, DNA viruses that cause both acute and persistent infections of susceptible hosts. The factors which determine whether the virus will be eliminated or whether a persistent infection will develop have not been defined in detail, although in the case of HBV, a central role of the immune response in eliminating infection is suggested by the observation that most infants infected at birth develop a persistent infection, while only 5–10% of adults when infected with the virus become persistently infected. The same age-related effect on the outcome of infection is seen in ducks, in which transmission of DHBV to newly hatched ducklings invariably results in the development of persistent infection, whilst infection of adult ducks is usually transient (Jilbert *et al.,* 1988, 1992).

Experimental transmission of DHBV to newly hatched ducklings has been used by ourselves and others to provide a reproducible *in vivo* system for quantitative studies of virus transmission and dissemination in susceptible hosts (Omata *et al.,* 1984; Tagawa *et al.,* 1985; Freiman *et al.,* 1988; Jilbert *et al.,* 1988; Marion *et al.,* 1987). In a previous study we found that following intravenous or intraperitoneal inoculation of 1-day-old ducks with high doses of DHBV ($7.5 \times 10^7$ DNA genome equivalents; sufficient to deliver virus to ~10% of liver cells), infection of the liver was first detected by the presence of a virus antigen and virus DNA in randomly scattered hepatocytes on Day 1–2 postinoculation (p.i.), and in the serum (virus DNA) on Day 3 p.i. (Jilbert *et al.,* 1988). Similarly, Marion *et al.* (1987) inoculated 2- to 5-day-old ducklings intramuscularly with $3 \times 10^7$ virus genome equivalents and first detected serum DHBV DNA Day 4 p.i.

In contrast, in adult humans HBV surface antigen (HBsAg) typically appeared in the serum 21–77 days after subcutaneous inoculation of virus, and clinical symptoms first appeared after HBsAg had been circulating for at least 21–66 days (Hoofnagle *et al.,* 1978). The incubation periods to both antigenaemia and clinical ill-

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ness were inversely related to virus dose (Barker and Murray, 1972). In neonates infected by vertical transmission, antigenaemia usually appeared 30–150 days after birth (Beasley and Stevens, 1978) but clinical disease was rare. These studies suggest a discrepancy in the incubation periods between naturally acquired HBV and experimental DHBV infections. We therefore examined the effect of DHBV dose on the incubation period of infection using as our inoculum, pooled serum from congenitally DHBV-infected ducks which contained $9.5 \times 10^9$ DHBV DNA genomes per milliliter. As in humans, the onset of viraemia was inversely related to the dose of inoculated virus. However, in contrast to results in humans, even inoculation with the equivalent of one virus DNA genome produced relatively short incubation periods with DHBV DNA and surface antigen detectable in the blood by Day 14 or 29 p.i.

To further explore the kinetics of infection after low dose inocula, we inoculated newly hatched ducks intravenously with sufficient DHBV to infect only ~0.0001% of total liver cells and then examined autopsy tissues harvested daily from Days 3 to 16 p.i. Infection was first detected at on Day 4 p.i. in ~0.035% of hepatocytes and spread finally to involve the entire hepatocyte population. The exponential increase in the percentage of infected cells from Day 4 to Day 14 p.i. suggested that there were no major delays in virus replication within the liver.

## MATERIALS AND METHODS

### Animals

DHBV-negative Pekin-Aylesbury cross-bred ducks and congenitally DHBV-infected Pekin ducks (*Anas domesticus platyrhynchos*) were obtained on the day of hatching from two independent commercial suppliers. DHBV-negative and -positive ducklings were housed in separate animal holding facilities. All animal handling protocols were reviewed and approved by the Institute of Medical and Veterinary Science (IMVS) and University of Adelaide Animal Ethics Committees and followed the Australian National Health and Medical Research Committee guidelines.

### Preparation of pooled serum

Three groups of 30 congenitally DHBV-infected ducks were anaesthetised with Nembutal and bled by cardiac puncture on Day 17 or Day 18 posthatch. Blood samples were held at room temperature for 6 hr and then at 4° overnight to allow clot formation before being spun at 2000 rpm at 4° for 10 min. Serum samples from each group of 30 ducks were collected and frozen at −80° before being thawed and pooled to yield a total volume of 520 ml. The pooled serum was then filter sterilised, aliquoted, frozen at −80°, and thawed immediately before use. Normal duck serum (NDS) was prepared in a similar manner from DHBV-negative ducklings by cardiac puncture and collection of serum.

### Analysis of serum samples for DHBV DNA and surface antigen

Twenty-microliter serum samples were digested with 2 mg/ml of pronase, 0.1% sodium dodecyl sulphate (SDS), 0.02 *M* Tris–HCl (pH 7.5), 0.15 *M* NaCl, and 0.01 *M* EDTA in a total volume of 400 $\mu$l at 37° for 2 hr. Alternatively, 1.8-ml serum samples were first diluted in an equal volume of TN (0.02 *M* Tris–HCl (pH 7.5), 0.15 *M* NaCl), and virus was spun to equilibrium in a 7-ml, 20–60% sucrose gradient with a 1-ml, 70% sucrose cushion in a SW41 rotor at 100,000 *g* for 17 hr at 4°. Fractions (0.5 ml) were collected from the bottom of the tube and digested with 2 mg/ml pronase as described above for 30 min at 37°. In each case the samples were then extracted with equal volumes of a mixture of phenol and chloroform (1:1), and the nucleic acids were precipitated with ethanol and subjected to electrophoresis in 1.5% agarose and Southern blot hybridisation (Wahl *et al.,* 1979) using a $^{32}$P-labelled DNA probe containing the entire DHBV genome. Serum samples and sucrose gradient fractions were also spotted directly onto nitrocellulose and hybridised to detect DHBV DNA as previously described (Jilbert *et al.,* 1987). DNA quantitation was performed by comparison with DNA standards using a Molecular Dynamics Phosphor Imager system.

Serum samples were also analysed for DHBV surface antigen (DHBsAg) by ELISA and quantitated in all assays with reference to standard curves constructed using DHBV-positive pooled serum diluted in 1/1000 and 1/7500 normal duck serum in PBS: The DHBsAg content of the pooled serum (50 $\mu$g/ml) was measured in ELISA by comparison to highly purified DHBsAg (purified from pooled serum on sucrose gradients) which was quantitated by SDS–PAGE using molecular weight standards of known concentration. Western blot analysis was used to confirm the position of the DHBsAg-specific bands. DHBsAg ELISAs were performed on serum samples which were diluted 1/1000 and 1/7500 in PBS and used to coat 96-well microtitre plates (Disposable Products, Adelaide) at 37° for 1 hr. Plates were then washed in 0.05% Tween 20 in PBS (PBS-T) and blocked with 5% skim milk, in PBS-T at 37° for 1 hr, before addition of mouse monoclonal anti-preS (1H.1; kindly donated by Dr. John Pugh and described in Pugh *et al.,* 1995) diluted 1/10,000 in 5% skim milk in PBS-T, and incubated at 37° for 1 hr. After washing, bound antibody was detected using horseradish peroxidase conjugated sheep anti-mouse Ig (1/4000; Amersham, Australia) in PBS-T and visualised with OPD substrate (Sigma). The reaction was stopped after 15 min in the dark by the addition of 0.83 *M* $H_2SO_4$ and OD (490 nm) was determined using a Dynatech plate reader.

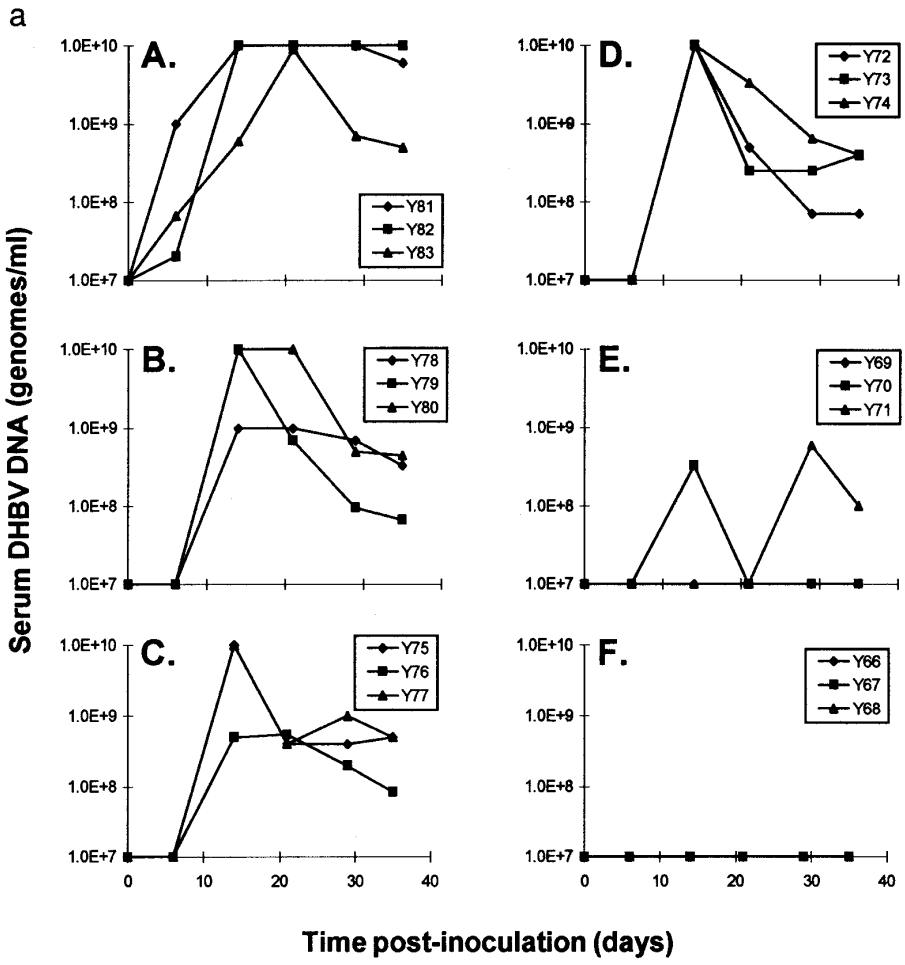


FIG. 1. Determination of the infectivity titre ($ID_{50}$) of DHBV-positive pooled serum in 3-day-old ducklings. Detection of DHBV DNA (a) and DHBsAg (b) in serum samples of groups of three ducklings inoculated intravenously with 100 $\mu$l of DHBV-positive pooled serum diluted from $10^{-5}$ (A), $10^{-6}$ (B), $10^{-7}$ (C), $10^{-8}$ (D), $10^{-9}$ (E), $10^{-10}$ (F). Serum samples collected on Days 6, 14, 21, 29, and 35 p.i. were analysed for DHBV DNA by spot blot hybridisation and for DHBsAg by ELISA as described under Materials and Methods. The infectivity titre of the pooled serum inoculum was calculated as $1.5 \times 10^{10}$ $ID_{50}$/ml. Thus the infectious dose delivered to each duck ranged from $1.5 \times 10^{4}$ (A) to $1.5 \times 10^{-1}$ (F) $ID_{50}$.

### DHBV antigen and DNA detection in tissues

Following euthanasia, 2- to 3-$mm^3$ pieces of liver tissue sampled from two different sites in each lobe and samples of spleen, pancreas, bursa, and kidney tissue were fixed in ethanol:acetic acid (EAA, 3:1) at room temperature for 30 min, followed by overnight fixation in 70% ethanol at 4°, before being processed into paraffin wax and sectioned at 6 $\mu$m onto gelatin-coated slides (Rogers, 1979). DHBsAg and DHBcAg were detected in EAA-fixed tissues by standard immunoperoxidase techniques using rabbit anti-recombinant DHBcAg (Jilbert *et al.,* 1987, 1992) or mouse monoclonal anti-preS (1H.1; Pugh *et al.,* 1995) followed by HRP-conjugated sheep anti-mouse Ig and diaminobenzidine.

DHBV DNA was detected by *in situ* hybridisation in EAA-fixed tissue sections (Jilbert *et al.,* 1987) using 2.5 ng/$\mu$l of digoxigenin–dUTP-labelled (DIG–dUTP; Boehringer Mannheim) DHBV DNA probes prepared by random primer labelling according to the manufacturer's instructions. Plasmid pUC19 DNA similarly labelled with DIG-dUTP was used to control the specificity of hybridisation. Visualisation of DIG-dUTP was performed according to the manufacturer's instructions by immunocytochemistry with anti-DIG-alkaline phosphatase followed by 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue tetrazolium salt (NBT). Following development, sections were counterstained with haematoxylin, dehydrated, washed in Histoclear 1, and mounted in Histomount (National Diagnostics, Atlanta). Total DNA was also purified from liver tissue and analysed for DHBV DNA by Southern blot hybridisation, exactly as described by Jilbert *et al.* (1992).

## RESULTS

### Comparison between $ID_{50}$ titre and virus genome copy number

All infection experiments were performed using one large pool of sera collected from 90 congenitally infected

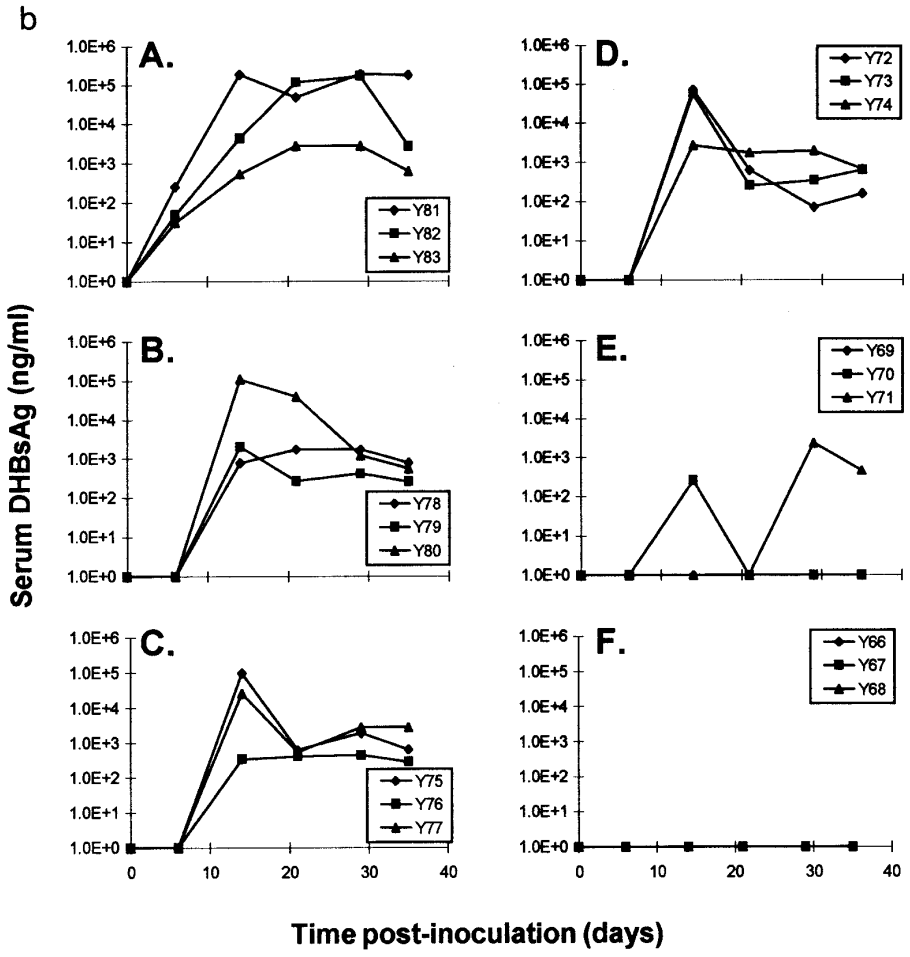


FIG. 1—*Continued*

ducks on Day 17 or Day 18 posthatch. The DHBV DNA content of this pool was quantitated in two ways: (1) Samples from the DHBV-positive pool were digested directly with pronase and SDS and then analysed by Southern and spot blot hybridisation; (2) virus from the DHBV-positive pool was first banded in 20–60% sucrose (final density 1.15–1.16 g/ml) before digestion and Southern and spot blot analysis as above. The DHBV DNA content of the pool was estimated by comparison to standard amounts of genomic length DHBV DNA excised from plasmid pSP.DHBV5.2Ga1×2, which served as an internal marker in all blots. Genome copy number was calculated from the relationship that 1 DHBV genome contains $3 \times 10^{-6}$ pg DNA. Both methods of quantitation showed good agreement, with estimates of $9.3 \times 10^9$ DHBV genomes/ml for DNA directly extracted from serum and $9.75 \times 10^9$ genomes/ml for material that was first banded in sucrose. These findings demonstrated that the vast majority of viral genomes present in the DHBV-positive pool were encapsidated into virions.

To determine the *in vivo* $ID_{50}$ titre of the above DHBV-positive pool, twenty-one 3-day-old DHBV-negative ducklings were prebled and inoculated intravenously with 100 $\mu$l of the pool diluted in 10-fold steps from $10^{-5}$ to $10^{-10}$ in NDS. Ducklings were bled on Days 6, 14, 21, 29, and 35 p.i. and serum samples were tested for DHBV DNA by spot blot hybridisation and for DHBsAg by ELISA. In all ducks the appearance of both markers coincided, allowing clear identification of infection. The DHBV-positive pool was calculated to contain $1.5 \times 10^{10}$ $ID_{50}$/ml using the Spearman–Kärber method (Finney, 1978) with 95% confidence intervals of $3.0 \times 10^9$ to $6.3 \times 10^{10}$ $ID_{50}$/ml. Thus the DHBV DNA content was within the 95% confidence intervals for the $ID_{50}$/ml indicating equivalence between one DHBV genome and one infectious unit within the limits of the assays.

## Relationship of incubation period to size of virus inoculum

Samples from the above experiment were then analysed in detail to define the onset of infection in ducks receiving different sized inocula (Figs. 1a and 1b). Viraemia was present on Day 6 p.i. (the first time point examined) in ducks receiving $1.5 \times 10^4$ $ID_{50}$ and was delayed to Day 14 in ducks receiving $1.5 \times 10^3$, $1.5 \times 10^2$, and

$1.5 \times 10^1$ $ID_{50}$. Of three ducks receiving 1.5 $ID_{50}$ inoculum, incubation periods of 14 and 29 days were seen in the two ducks that became infected. Relative levels of serum DHBsAg in each sample closely reflected viral DNA levels (Figs. 1a and 1b). Once viraemia was established, viral DNA persisted in all ducks except duck Y70 at levels ranging from $2 \times 10^7$ to $1 \times 10^{10}$ DHBV DNA genomes/ml, independently of initial inoculum size. Southern blot analysis and immunoperoxidase staining of DHBcAg in autopsy liver tissue collected on Day 43 p.i. demonstrated that infection had persisted to Day 43 in all ducks initially infected. Furthermore there was direct correlation between markers of infection in the serum and in the liver in all ducks at all time points with the exception of duck Y70, which received 1.5 $ID_{50}$ and showed transient viraemia but persistent infection in the liver.

## Kinetics of spread of infection following low dose inoculation

Twenty-eight DHBV-negative ducklings were prebled on Day 4 posthatching and inoculated intravenously with 100 $\mu$l of DHBV-positive pool diluted to $10^{-6}$ in NDS, i.e., containing $1.5 \times 10^3$ $ID_{50}$. Thus, this dilution would reliably produce viraemia by Day 14 p.i. (Figs. 1a and 1b) whilst at the same time allowing us to study the spread of infection from a low percentage of infected cells. Two additional ducks which were inoculated with NDS alone and held in the same pen as those ducks which received DHBV were negative for all markers of DHBV infection throughout the experiment and served as controls for horizontal transmission of virus.

Following inoculation, one group of seven ducks which had been inoculated with the DHBV-positive pool and the two ducks which received NDS alone were bled every second day and serum samples were assessed for the presence of DHBV DNA by spot blot hybridisation and for DHBsAg by ELISA (Figs. 2A and 2B). The onset of viraemia in inoculated ducks varied between 10 and 15 days p.i., being first detected in four of the seven ducks on Day 10 p.i.

All 28 ducks which had been inoculated with DHBV were then used to assess the extent of virus infection in liver and other tissues. Each day from Day 3 to Day 16 p.i., 2 ducks were sacrificed and assessed for the presence of DHBV DNA by Southern blot hybridisation of extracted liver (Table 1), for *in situ* hybridisation in sections of liver tissue (data not shown), and for viral antigen expression in liver (Fig. 3), bursa, spleen, kidney, and pancreas (Table 1). DHBcAg and DHBsAg expression and cytoplasmic DHBV DNA were first seen in 0.035% of hepatocytes in the liver of 1 of the 2 ducks on Day 4 p.i. and in each duck autopsied from Day 5 to Day 7 p.i. (Table 1, Fig. 2C) as single cells, pairs, or small groups of DHBcAg, DHBsAg, and DHBV DNA-positive hepatocytes (Fig. 3). Exponential increases in the percentage of infected cells occurred from Day 4 to Day 14 p.i. with a mean doubling time of 16 hr (Fig. 2C). By Day 14 p.i. replication was seen in >95% of hepatocytes. In contrast, DHBV DNA was not detected by Southern blot hybridisation in liver homogenates until Day 8 p.i. (Table 1), significantly later than the detection of low numbers of DHBV-positive cells in tissue sections.

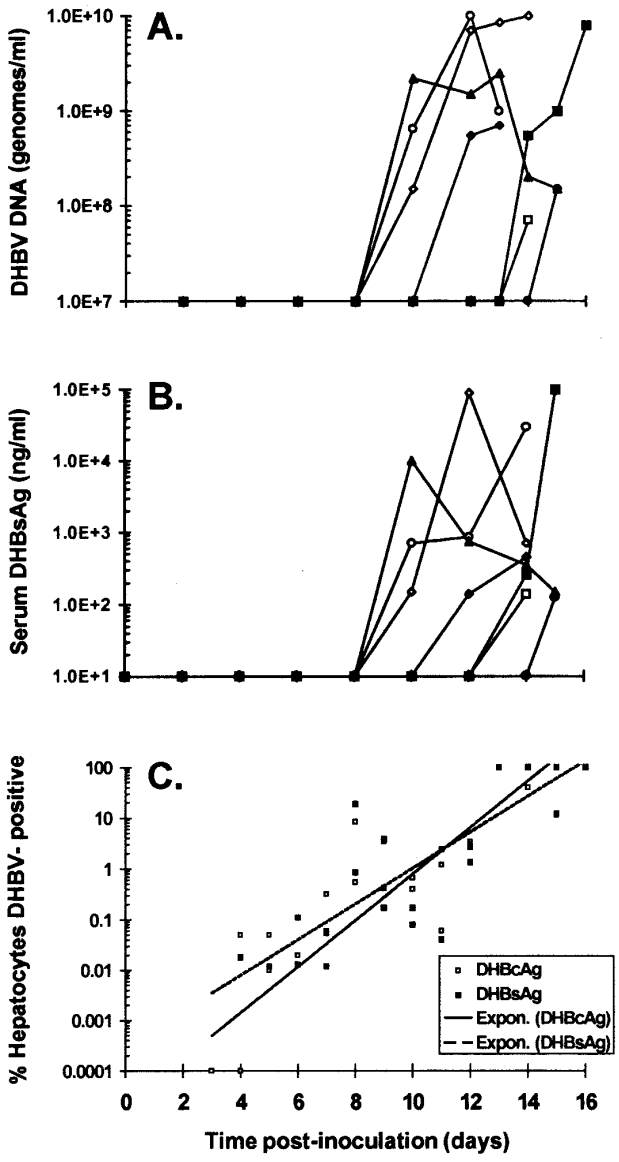


FIG. 2. The time course of DHBV infection in a group of 28 4-day-old ducklings inoculated intravenously with DHBV-positive pooled serum diluted to $10^{-6}$ in NDS, i.e., containing $1.5 \times 10^3$ $ID_{50}$ doses of DHBV. A group of 7 ducks were bled every second day and serum samples were analysed for (A) DHBV DNA by spot blot hybridisation and (B) DHBsAg by ELISA. (C) DHBV infection was also monitored in all 28 ducks from Days 3 to 16 p.i. by immunoperoxidase staining of DHBcAg and DHBsAg in sections of EAA-fixed autopsy liver tissue. The percentage of DHBcAg- and DHBsAg-positive hepatocytes was determined by cell counts in sections counterstained with haematoxylin and the calculated line of best fit for exponential increase in the number of DHBcAg- and DHBsAg-positive hepatocytes is shown.

Expression of DHBcAg, DHBsAg, and DHBV DNA in

TABLE 1

Time Course of DHBV Infection in Ducks Receiving $1.5 \times 10^3$ $ID_{50}$ of DHBV

| Duck[a] | Day p.i. | Serum DHBV DNA[b] | DHBV DNA (gen/liver cell)[c] | % Hepatocytes DHBcAg-positive[d] | % Hepatocytes DHBsAg-positive[d] |
|---|---|---|---|---|---|
| W62 | 3 | — | <1.0 | <0.01 | <0.01 |
| W63 | 3 | — | <1.0 | <0.01 | <0.01 |
| W64 | 4 | — | <1.0 | <0.01 | <0.01 |
| W65 | 4 | — | <1.0 | 0.05 | 0.02 |
| W66 | 5 | — | <1.0 | 0.05 | 0.01 |
| W67 | 5 | — | <1.0 | 0.01 | 0.02 |
| W68 | 6 | — | <1.0 | 0.02 | 0.01 |
| W69 | 6 | — | <1.0 | 0.02 | 0.11 |
| W70 | 7 | — | <1.0 | 0.32 | 0.05 |
| W71 | 7 | — | <1.0 | 0.06 | 0.01 |
| W72 | 8 | — | 3.6 | 0.54 | 0.85 |
| W73 | 8 | $2 \times 10^7$ | 24.2 | 8.4 | 18.7 (K)[d] |
| W74 | 9 | — | 14.0 | 3.5 | 3.84 |
| W75 | 9 | — | 3.3 | 0.42 | 0.17 |
| W77 | 10 | — | 1.2 | 0.67 | 0.17 |
| W78 | 10 | — | <1.0 | 0.4 | 0.08 |
| W79 | 11 | — | <1.0 | 0.06 | 0.04 |
| W80 | 11 | — | 5.8 | 1.2 | 2.42 |
| W81 | 12 | — | 9.1 | 3.4 | 1.34 |
| W82 | 12 | — | 6.0 | 3.0 | 2.7 |
| B62 | 13 | $7 \times 10^8$ | 99.2 | >95 (S)[d] | >95 (K)[d] |
| B63 | 13 | $1 \times 10^9$ | 79.4 | >95 (K, S, P)[d] | >95 (K, S)[d] |
| B64 | 14 | $1 \times 10^{10}$ | 118.7 | >95 (K, S, P)[d] | >95 (P)[d] |
| B65 | 14 | $7 \times 10^7$ | 19.9 | 39.3 | >95 (K)[d] |
| B66 | 15 | $1.5 \times 10^8$ | 57.6 | >95 (K, S. P)[d] | >95 (K, P)[d] |
| B67 | 15 | $1.5 \times 10^8$ | 19.2 | 11.4 | 12.2 (K)[d] |
| B68 | 16 | $8 \times 10^9$ | 75.6 | >95 | >95 (K, S)[d] |
| B69 (NDS)[e] | 16 | — | <1.0 | <0.01 | <0.01 |
| B70 (NDS) | 16 | — | <1.0 | <0.01 | <0.01 |

[a] Four-day-old ducklings were inoculated intravenously with 100 $\mu$l of DHBV-positive pooled serum diluted to $10^{-6}$ in NDS, i.e., containing $1.5 \times 10^3$ $ID_{50}$ of DHBV.

[b] Serum DHBV DNA was determined by spot blot hybridisation with a minimum level of detection of $1 \times 10^7$ DHBV DNA genomes/ml.

[c] The DHBV DNA content (DHBV DNA genome equivalents per liver cell) was determined by Southern blot hybridisation of 16 $\mu$g of total cellular DNA extracted from 9 mg (equivalent to $6.3 \times 10^6$ total liver cells) of liver using cloned plasmid DNA as control, assuming that each DHBV DNA genome is $3 \times 10^{-6}$ pg.

[d] DHBsAg and DHBcAg were detected in EAA-fixed tissue using standard immunoperoxidase techniques. Expression of DHBcAg and DHBsAg was seen where indicated by kidney (K), spleen (S), and pancreas (P).

[e] Ducks B69 and B70 received NDS alone without the addition of DHBV.

extrahepatic tissues was not seen until Day 13 p.i. in spleen (germinal centres), pancreas (acinar cells), and kidney (glomeruli), while DHBcAg, DHBsAg, and DHBV DNA were not detected in bursa at any time point.

## DISCUSSION

The finding of approximate equivalence between the number of DHBV genomes banding at a density of 1.15–1.16 g/ml and the $ID_{50}$ titre in pooled serum from 17- to 18-day-old congenitally infected ducklings carries important implications. This demonstrated that the DHBV-positive pool did not contain significant amounts of defective genomes, e.g., as might arise by point mutation or subgenomic deletion. Naturally occurring mutants of HBV, involving either base substitutions or deletions, have been implicated in human in mechanisms of virus persistence, in escape from vaccine-induced immunity, in altered virulence, and as a possible cause of one form of non-A–E hepatitis. However, it has not usually been possible to directly examine the replication competence (or virulence) of the different mutant sequences that are found. Although the sequence diversity within the virus stock was not examined in this study, our results indicate that, at least up to 17–18 days of age after congenital infection, full replication competence was maintained in all those genomes that were encapsidated and exported. Further studies are needed to define more fully the spectrum of genetic diversity that can be tolerated in long-standing infection with a stable, slowly replicating reser-

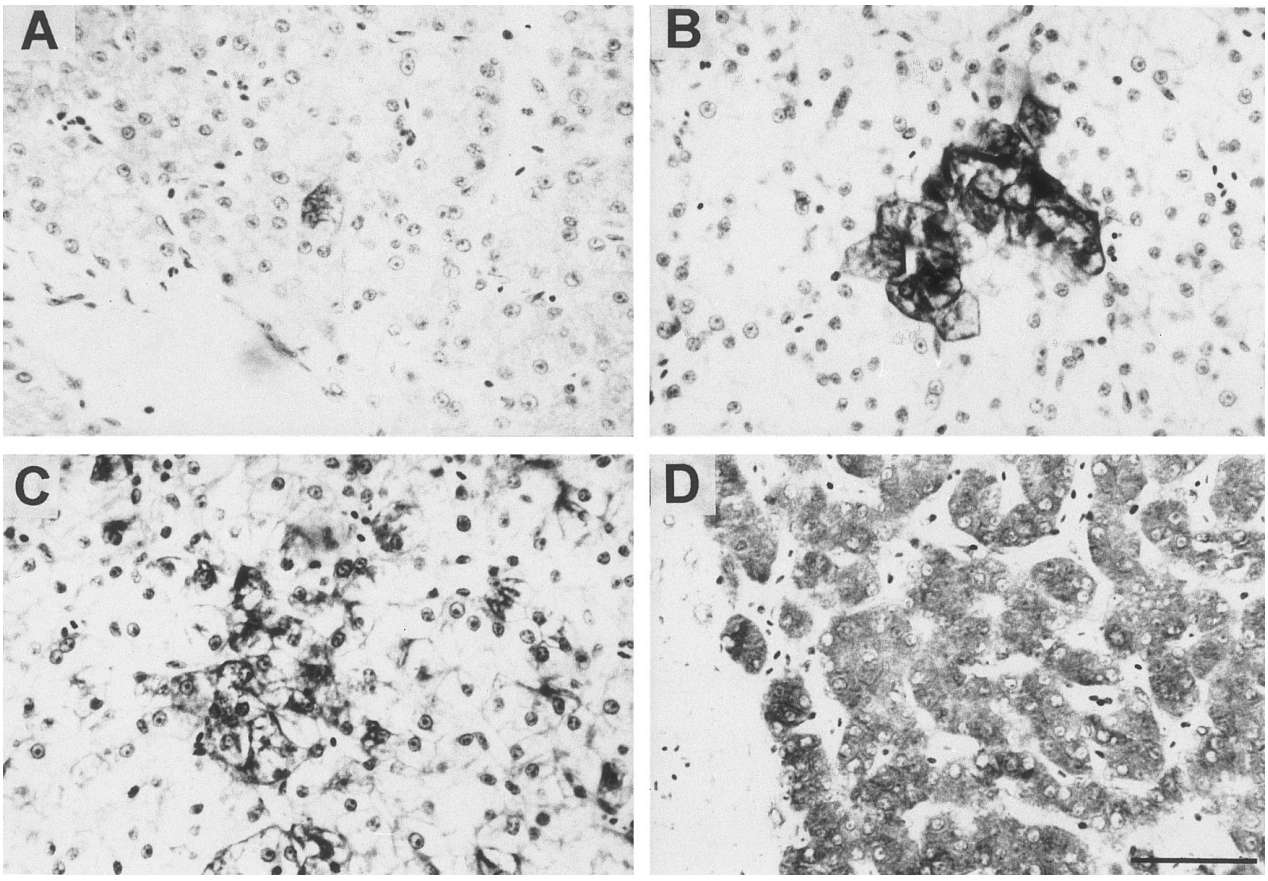


FIG. 3. Detection of DHBsAg-positive hepatocytes in the liver of ducklings which received $1.5 \times 10^3$ $ID_{50}$ of DHBV. Sections of EAA-fixed autopsy liver tissue from ducks sacrificed on: (A) Day 5 (duck W67, 0.02% hepatocytes DHBsAg-positive); (B) Day 8 (duck W72, 0.852% hepatocytes DHBsAg-positive); (C) Day 8 (duck W73, 18.2% hepatocytes DHBsAg-positive); and (D) Day 13 p.i. (duck B62, >95% hepatocytes DHBsAg-positive). DHBsAg was detected by immunoperoxidase staining with mouse monoclonal anti-preS antibodies (1H.1; Pugh *et al.,* 1995). Nuclei were counterstained with haematoxylin. Bar = 50 $\mu$m.

voir of covalently closed circular DNA and to document the pathogenetic consequences of the various mutants that may arise. In addition, the close concordance between relative DHBV DNA and DHBsAg levels in the various samples indicated that, at least in the stage of infection examined in this study, the ratio between whole virions and empty DHBsAg particles did not undergo any systematic change.

In this study, the incubation period to onset of viraemia was prolonged with very low dose inocula, but never beyond 29 days, in contrast to HBV in adult humans in which the appearance of circulating HBsAg after low dose inocula ($10^{-7}$) was delayed to greater than 90 days (Barker and Murray, 1972). This finding reinforces the view that neonatal duck hepatocytes are highly permissive to infection, that immune responses do not play a major role in suppressing infection at this age, and that once infection is initiated even by one virion, it rapidly progresses to involve all susceptible cells. Among ducks inoculated intravenously with $1.5 \times 10^3$ $ID_{50}$ (conditions analogous to infection in humans through needle-sharing), limited bird-to-bird variation was seen and all ducks showed widespread infection of hepatocytes by Day 13 p.i. Assuming that most of the inoculated virus reached the liver, this would be sufficient to infect ~0.0001% total liver cells ($7 \times 10^8$ cells per gram of duck liver; Jilbert *et al.,* 1992). Following replication in susceptible hepatocytes newly produced virus may in theory spread to neighbouring cells by direct cell-to-cell transfer or diffusion via the space of Dissé, or it may pass the sinusoidal lining cell barrier, enter the bloodstream, and infect more distant cells. The findings of our study are consistent with virus spread by a combination of these routes as we observe DHBV-infected cells during the exponential phase of infection as single cells, pairs, and small groups of cells. We assume that infected cells are present in the liver on Days 1–3 p.i. but at levels below the limit of detection of the immunoperoxidase screening procedure (sensitivity 0.005–0.01% of hepatocytes). Thus, commencing with approximately ~0.0001% of hepatocytes being initially infected, infection would not reach detectable levels until a number of rounds of infection had occurred. The percentage of DHBV-positive hepatocytes increased exponentially throughout the early phase of

infection with a mean doubling time of 16 hr (Fig. 2C). Increases in the percentage of infected hepatocytes from 0.0001 to 0.035% could therefore be realistically achieved by Days 4–5 p.i.

The above findings can be compared with mousepox (ectromelia) infection in mice. Following footpad inoculation of $5 \times 10^2$ $ID_{50}$ of ectromelia, virus replication in the liver occurred exponentially from Days 3–5 p.i. reaching levels of $10^9$–$10^{10}$ $ID_{50}$ per gram in fatal cases, but reaching a plateau and then falling rapidly with the appearance of antibody in nonfatal cases. During the exponential phase the mean doubling time for infectious particles in the liver was 3.7 hr (Fenner, 1949). In contrast, as described in the current study of low dose intravenous inoculation of DHBV, infection in the liver achieved a maximal DHBV DNA content and percentage of infected cells only by Days 13–14 p.i. with a mean doubling time in the percentage of infected cells of 16 hr. In a previous study using high dose incocula (Jilbert *et al.,* 1988), the average DHBV DNA content per microgram of liver DNA rose exponentially from Days 2–4 p.i. with a doubling time of 9.4 hr. By either measure, it is clear that the rate of replication after DHBV gained access to the liver was markedly slower than that reported for ectromelia and therefore that slower intracellular replication kinetics could provide at least one of the reasons for the slower development of infection in DHBV-infected ducks.

Using similar methods of detection, virus replication in extrahepatic tissues was seen only considerably later than in the liver. This might be because virus replication is inherently slower in cells of nonhepatic origin, or because other organs might lack efficient pathways for uptake of virus particles from the circulation. In any event, the extrahepatic sites studied clearly play a less important role than the liver in the progression of infection at this stage.

These studies form a basis for examination of the effects of age and route of inoculation on pathogenesis and also for the study of host/virus relationships and the emergence of immune escape mutants in hepadnavirus infection.

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